

TCF/LEF dependent and independent regulation of Wnt/ β -catenin transcription

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Review timeline:

Submission date:	19th Dec 2017
Editorial Decision:	2nd Feb 2018
Revision received:	8th Jul 2018
Editorial Decision:	3rd Aug 2018
Revision received:	29th Aug 2018
Editorial Decision:	6th Sep 2018
Revision Received:	19th Sep 2018
Accepted:	28th Sep 2018

Editor: Deniz Senyilmaz Tiebe / Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

2nd Feb 2018

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you can see from the comments, all three referees express interest in the proposed TCF-independent mode of β -catenin-mediated transcription regulation. However, they also raise substantive concerns with the analysis that would need to be addressed before they can support publication here. Based on the overall interest expressed in the reports, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three reviewers, particularly focusing on the following points:

1. Improve data analysis and presentation as requested by referees #1 and #3,
2. Provide the controls for β -catenin ChIP (referee #1), TCF knockdown analysis (referee #2) and RNA-seq analysis (referee #3)
3. Provide further evidence that the observed TCF-independent β -catenin response is mediated by β -catenin-dependent transcription (referee #2)
4. Address the issue of the relevance of TCF-independent role of β -catenin (referees #2 and #3)
5. Add further analysis of the role of FOXO/ β -catenin interaction in TCF-independent gene regulation, as requested by all reviewers.

REFeree REPORTS:

Referee #1:

This manuscript addresses an important question in canonical Wnt signaling: what percentage of Wnt transcriptional targets are dependent on the TCF family of transcription factors (TFs)? There are many reports of TFs besides TCFs regulating Wnt targets, but these reports are largely based on

overexpression of TFs and beta-catenin using reporter genes to monitor transcription. There is also a report from the Clevers lab claiming that the vast majority of Wnt targets in colorectal cell lines are TCF dependent, based on beta-catenin ChIPseq with or without a TCF dominant negative.

In this report, the authors perform the impressive feat of knocking out all four TCF genes in HEK293T cells, as well as knocking out beta-catenin and even creating lines lacking TCFs and beta-catenin. These lines were then stimulated with a GSK3 inhibitor to activate Wnt targets and characterized using RNAseq. ChIPseq of beta-catenin in control and TCF mutant lines were also performed.

These experiments provide some interesting findings. While the majority of beta-catenin dependent CHIR regulated genes (~85%) are TCF-dependent, there are many genes that are regulated by beta-catenin independently of TCFs. This is perhaps not surprising given the evidence for TCF and non-TCFs in Wnt gene regulation but it is very satisfying to see the problem addressed in such a systematic and rigorous manner. In addition, the authors make the surprising finding that in cells lacking TCFs, stabilization of beta-catenin (via CHIR) regulates a large group of additional genes. Redirection of beta-catenin activity in the absence of TCFs is further supported by beta-catenin ChIP-seq in TCF mutant lines. Some evidence that this "Ghost" activity of beta-catenin is mediated in part by FOXO proteins is also provided.

There are several deficiencies in the current manuscript that I believe should be addressed, outlined below.

1) While the figures and illustrations provided help the reader to understand the complexities of the data, more detailed summaries of the transcriptome and ChIPseq results should be provided in excel spreadsheets in the supplemental portion of the manuscript. For the RNAseq data, the relative values for each of the genes whose expression is altered upon CHIR treatment in WT and mutant cells should be provided. The reader should be able to use these spreadsheets to easily acquire the whole set of 139 genes that are regulated in a TCF and beta-catenin manner (and know how many are activated or inhibited by CHIR). Same for the other classes of genes described. For the ChIPseq data in WT and TCF mutant cells, the position of the beta-catenin peaks should be indicated, along with the nearest 5' and 3' gene (please use the most current reference genome).

2) In regard to the ~3900 beta-catenin peaks referred to in the manuscript, are there any controls to determine whether they are truly due to beta-catenin, e.g., ChIPseq in the absence of CHIR or in a beta-catenin mutant? It seems relevant to question the cleanliness of the beta-catenin antibody, given the fact that there are 27 of 166 genes that are regulated by CHIR/beta-catenin independently of TCFs, yet only 67 of the ~2300 beta-catenin peaks appear to be independent of TCFs.

3) I was frustrated that the RNAseq and ChIPseq data in the manuscript were not better integrated. How many of the 166 CHIR regulated beta-catenin dependent genes in WT cells have a ChIPseq peak near them? Are any of the 67 TCF-independent ChIPseq peaks near genes that are regulated by beta-catenin but not TCF? Same for the 134 "ghost" targets and the 1600 new ChIPseq peaks. The authors should provide this data in table or figure form in the main body of the manuscript.

4) Another key question that the authors' data should address is the percentage of Wnt targets in HEK293T cells that are subject to TCF repression in the absence of CHIR, i.e., are these genes expressed at higher levels in TCF mutants compared to WT? The absence of this analysis is puzzling, given that the Basler lab has recently published a nice paper addressing this question in *Drosophila* cultured cells. It would be valuable to examine this in HEK293T cells as well.

5) I have several issues with the way the manuscript is currently written:

a) I really don't like the title. What about something a little less dramatic, e.g., TCF dependent and independent regulation of Wnt/beta-catenin transcription. I think this title encompasses the entire data of the manuscript better than the current version.

b) I strongly object to the statement that "the central tenet" of the Wnt pathway is exclusive regulation of targets by TCFs. This statement needs to be toned down. Many, many Wnt researchers are open to the possibility that some Wnt targets are regulated by non-TCFs.

c) page 3: I don't like when researchers exclude GSK3a from the Wnt pathway. CHIR inhibits both GSK3a and GSK3b, and both kinases need to be mutated to see strong Wnt gain of function phenotypes in mammals. Just use GSK3 and you can help to correct the wide misconception that GSK3b is the more important kinase, or direct me to data that support that claim, other than the fact that everyone repeats the same mistake.

d) page 6: the first paragraph oversimplifies the debate over TCF/non-TCF mediation of Wnt gene regulation in the literature. Provide a more balanced view.

e) page 7: Axin2 is "a" prototypical target, not "the"

f) page 8: change "In our set up" to "Under our conditions"

g) page 9, line 2: I don't like the use of the term "direct" when "beta-catenin dependent would suffice. Direct implies a direct biochemical connection in the view of many researchers.

Referee #2:

This is a well-written and straightforward MS offering evidence that b-catenin can regulate gene expression in absence of TCF/LEF DNA binding platforms.

I see few main issues:

General. I really do not like the title and naming of this phenomenon as Ghost... It does not add anything just confusion. B-catenin GHOST makes me think of a real/(i.e.)identified GHOST TF with that name, that of course is different from the enigmatic GHOST(s?) phenomenon by which b-catenin operates as the authors are hinting. As external example, the Wnt-STOP signaling is different because it is an acronym (STabilization Of Protein).

Just call this differently: TCF/LEF independent transcription or similar statements would suffice.

Specific points

1) A key missing experiment is the use of clones of the TCF/LEF CRISPR generated clones (d4TCF) reconstituted with at least 1 of these factors.

There is currently no rescue experiment. The main problem here is very technical: they are subcloning and subcloning and subcloning their 293 cells in order to make such 4x KO reagent (that might be potentially very useful to the community, no doubt, if perfectly controlled). The risk, however, is that by constantly cloning they may select for specific cell sub-populations of the original 293 culture (or in fact for descendants of 1 single cell) that could have ab initio some genetic or epigenetic changes responsible for the observed changes in gene expression/responsiveness etc that are admittedly quite low in numbers, as developed in point 2 below. ; and/or generate new cellular species by simple genetic drifting while culturing. And the control is parental 293 cells (where the heterogeneity is diluted in the whole population "average", or other clones of 293 that may represent a world on their own... These are due controls, but not internal perfect controls

2) The second main problem for me is in the numbers. Upon CHIR-GSK3 inhibition (as proxy of Wnt stimulation), B-catenin controls 231 genes (81 Up and 150 Downregulated). In absence of TCFs, only 4 genes are up (and upregulation by nuclear b-catenin is by far the most understood phenomenon). 23 genes are downregulated without TCF. What to make of all those repressed genes is unclear, as this is hard to rationalize. it can be a direct effect or an indirect effect caused by the lack of a positive target of b-catenin. This is to say that there are many reasons for changing gene expression. And, irrespectively, 4 genes out of 81 is a mere 5%. Are these genes at least directly binding b-catenin in their cis-regulatory promoter or enhancer elements?

Again the problem behind my questioning is certainly not raising silly doubts on undefined alternative hypotheses but it is quite cogent to the point at stake: are we truly observing a b-catenin induced transcription independent of TCF, or, rather, an indirect effect caused by whatever functions b-catenin may have in the cytoplasm? or due to the fact that b-catenin is anyway part of the destruction complex (that work on several other proteins beyond b-catenin in Wnt-off conditions, see Wnt STOP and other related stories), and thus in presence of CHIR+b-catenin KD still many

proteins may get stabilized leading to some changes in gene expression down the road. Please also note (page 11) that 40% of genes modulated by CHIR are neither TCF nor b-catenin dependent, which is very interesting but just to say that there can be a broad array of fluctuations (and technical/biological intersection thereof) that may explain 5% of differential gene-expression changes.

3) Connected to the above, of course I also appreciate (page 10) that, moving from gene expression to genome occupancy, they also find "1600 statistically significant new enriched regions (i.e. occupied by -catenin) in d4CTF cells (Figure 5a)."

This gives me confidence that what they are describing may be real. Only that I find the story still preliminary at this stage, requiring some reinforcements and better controls.

What about the FOXO connection:

a) is B-catenin binding to FOXO?

b) Can they try chip-rechip with b-catenin and foxo antibodies?

4) Are these FOXO sites within or adjoining the TCF motifs? and for how many loci? if not, How to explain the fact that this FOXO -responses and in general this TCF-independent phenomenon is, in truth, only detected in absence of TCF (and not just irrespectively of)

5) an interesting finding is where they describe that b-catenin is still bound to TCF sites in absence of tcf lef proteins. Fig4. They write that

Curiously, motif analysis of these 67 peaks shows a significant presence of TCF4 and TCF3 consensus binding sequences,

This is all based on motif finder predictions, which is OK but do they know, by ChipSeq or ChipPCR, if all or a group of these genes is in fact really bound in the control parental cells by TCFs?

If so, this would make a stronger case in favor of another set of TFs able to bind and recruit b-catenin that are not TCFs, but may perhaps compete with TCF in normal conditions, new forms of default repressions etc. and other interesting ideas (to be discussed, perhaps).

Referee #3:

Comments on EMBOJ-2017-98873: "Activated β catenin Acquires a GHOST Transcriptional Activity in the Absence of TCF/LEF Transcription Factors" by Doumpas and colleagues.

This study addresses the question whether β -catenin-dependent transcriptional responses of the canonical Wnt pathway are exclusively mediated by TCF/LEF proteins. To this end, the authors generate clonal derivatives of HEK293 cells that simultaneously lack expression of the four TCF/LEF family members, of β -catenin, and of all of these factors. The knockout cells are used for comparative transcriptome analyses in response to a Wnt substitute, the GSK3 inhibitor CHIR99021. In addition, TCF/LEF-dependent and TCF/LEF-independent chromosomal distribution of β -catenin is determined. Based on their results the authors conclude that the vast majority of β -catenin-dependent transcriptional changes is TCF/LEF dependent - a confirmatory conclusion that had been reached already in previous studies from the same lab as well as from the Clevers group, the latter even using the same cell line. What the authors particularly stress is their observation of β -catenin-dependent gene expression changes that become discernible only in the complete absence of TCF/LEF proteins. The authors call this the β -catenin GHOST response. Overall, this is probably the technically most advanced, comprehensive, and unequivocal approach to tackle the biological question asked. The experiments are elegant and well performed. However, in view of existing knowledge, the findings presented are neither surprising nor completely novel. Competition among transcription factors for β -catenin is well known and the authors cite several papers that describe this phenomenon. Aside from this, I think the choice of HEK293 cells and the GSK3 inhibitor are major shortcomings of the study and have a strong negative impact on the physiological relevance of the findings. Moreover, the identification and characterization of the β -catenin-GHOST response genes is not sufficiently elaborated to be convincing and to allow accurate assessment of their significance. In my opinion, this piece of work employs a single, highly artificial experimental system to reveal a phenomenon that may actually exist but in view of the limitations of the study it remains obscure whether the so called β -catenin GHOST response ever plays a role in an authentic Wnt pathway response let alone a living organism.

Specific comments:

Major issues:

1. GSK3 is involved in many cellular processes and does not act exclusively in the canonical Wnt pathway. Therefore, it is not clear whether authors measure genuine Wnt pathway responses upon treatment with CHIR. Stimulation with Wnt growth factors should have been performed.
2. Although widely used as a test tube to study Wnt signalling mechanisms, it is not clear whether HEK293 cells can activate a physiologically relevant gene expression program in response to Wnt pathway activation. Likewise, it is completely unclear whether HEK293 cells are equipped to trigger meaningful TCF/LEF-independent gene expression. For this, relevant transcription factors would have to be expressed at sufficient levels. In view of this, the observations made could be an artefact of the HEK293 cell system and the importance of the conclusions is questionable. In support of this concern, the number of genes whose expression changes under the various experimental conditions seems rather low. The principle component analysis and the hierarchical clustering further suggest that differences among the cells arise from genotype not treatment.
3. The authors hypothesize that activation of the β -catenin-GHOST response relies on the interaction with low affinity transcription factor binding partners and becomes visible only in the absence of TCF/LEF proteins or when their interaction with β -catenin is prevented. There is no experimental evidence for this model. Again, the authors may be misled by the specific conditions in HEK293 cells. In fact, in physiologically relevant models, relative expression levels of TCF/LEF and non-TCF/LEF binding partners with similar affinities for β -catenin could even allow for co-existence of TCF/LEF-dependent and independent gene expression. Thus, there is no justification for invoking low affinity interaction and the need of TCF/LEF absence or inhibition.
4. The authors claim that 15 β -catenin-GHOST response genes were analysed but data are shown only for 9 genes, all of which are β -catenin-dependent. How do the authors arrive at a quote of 60% β -catenin-dependent genes?
5. How can there be β -catenin-independent genes among this group which according to the scheme in Figure 5b by definition should be β -catenin-dependent? This hints at a rather high false discovery rate in the RNA-seq analysis.
6. Is the 60% fraction representative for the entire β -catenin-GHOST response gene set? If so, the β -catenin-GHOST response gene set would be considerably smaller than 107. Accordingly, what is the true size of the β -catenin-GHOST response gene set, i. e. genes inducible by CHIR in dTCF4 cells and β -catenin-dependent?
7. How do the five genes analysed in Suppl. Figure 6 fit into the picture? Are they among the 107 potential β -catenin-GHOST response genes? The legend to Suppl. Figure 6 states that these genes are induced by CHIR in dTCF4 cells. Aside from the fact that the corresponding bars are hard to make out it appears to me that ARC2 and TVP23C do not show any kind of significant regulation. Again, are these false positives in the RNA-seq data set?
8. The authors insinuate that the β -catenin-GHOST response genes are regulated by β -catenin in non-TCF transcription factor complexes. This model could easily be corroborated (or refuted) by intersecting the true β -catenin-GHOST response genes with the β -catenin ChIP-seq data. Are confirmed β -catenin-GHOST response genes associated with β -catenin ChIP-seq peaks?
9. As an extension of this, genome browser views of examples for newly acquired β -catenin ChIP-seq peaks in d4TCF cells need to be shown.
10. The manuscript does not contain sufficient information about ChIP-seq peak location and differential gene expression under the different experimental conditions. The authors need to include supplementary tables listing ChIP-seq peaks and differentially expressed genes. This is especially important in relation to Figure 5b. A complete list of the 196 CHIR-responsive genes in d4TCF cells, the 27 CHIR-regulated but TCF-independent genes, and the β -catenin-GHOST response genes must be provided. In addition, RNA-seq and ChIP-seq data-sets should be made available to the scientific community by deposition in public repositories.
11. The authors present FOXO4 as an already known interaction partner of β -catenin that might contribute to the β -catenin-GHOST response. To make this point stronger and more convincing the authors should provide evidence that endogenous FOXO4 is expressed in HEK293 cells. On top of that it needs to be examined whether FOXO4 loss-of-function indeed affects the β -catenin-GHOST response.

Minor issues:

12. Figure 1: Please add Mw standard sizes to panel Fig. 1b; make spelling dbcat / dBcat consistent in panels Fig. 1b and c.
13. Figures 1d; 2e; 3b,c; 4c,d; Suppl. Figures 2, 3, 5, 6, 7: please add tick marks to y-axes.
14. Figure 2e, Suppl. Figure 5: it would be more informative and allow for better comparison of

genotype-dependent gene expression changes if the authors consistently applied the same experimental setup as used in Figure 1 d, i. e. measure FUT1 and HMOX1 also in WT/dBcat and in WT/d4TCF cells, respectively.

15. Figure 3: To allow for full assessment of β -catenin and TCF/LEF dependent/independent gene expression changes please include dBcat cells in the analyses of CHAC1, SNHG7, ADAMTS18, TERC, etc.):

16. Figure 4: Please correct the fatal spelling errors Tef3 > Tcf3; Tef4 > Tcf4; Het116 > Hct116.

17. Page 10: The authors mention that the 67 peaks which are occupied by β -catenin independently from TCF/LEF proteins show enrichment of binding motifs for HOXC/HOXD and MYB proteins and refer the reader to Figure 4d. However, this information is not included in the figure and consequently needs to be added.

17. Figure 5b: The Venn diagram does not properly reflect the numbers of genes whose regulation is specific and common to the two genotypes and needs to be corrected.

18. Suppl. Figure 6: The x-axis labels are incomplete; they do not match the numbers of bars and do not allow to identify treatment conditions.

1st Revision - authors' response

8th Jul 2018

We are pleased to re-submit our revised manuscript, now entitled “TCF/LEF dependent and independent regulation of Wnt/b-catenin transcription” for your consideration. We have addressed all reviewers' comments (see detailed response below) with substantial alterations of the original text and figures as well as with new experiments and analyses where appropriate. Specifically, we have:

1. Performed additional ChIP-seq experiments – we used different antibodies and additional control samples such as cells lacking b-catenin - which allowed us to exclude potential false positives (see new Figure 4 and Supplementary Figure 6)
2. Improved data analysis and presentation throughout the manuscript
3. Addressed the reproducibility of the clonal generation of the knockout cell lines
4. Performed rescue experiments of all as well as individually transfected TCF/LEF factors, and
5. Provided additional evidence supporting the role of FOXO4 in the TCF-independent gene regulation by motif analysis, protein-protein interaction between FOXO4 and b-catenin, and siRNA-mediated downregulation studies (new Figure 4e,f and 5e,f).

We are confident that implementing the criticisms and suggestions of the reviewers has improved both the clarity of our article and the strength of our findings.

DETAILED RESPONSE TO THE REVIEWERS

We are grateful to the Referees for evaluating our data so carefully. Integrating their comments helped us to significantly improve the strength and clarity of the manuscript. Below we detail how we have addressed each point (reviewers' comments are in red, and our responses are in black). For simplicity, we have also marked in red the parts, within the revised manuscript, in which we implemented the changes suggested.

Response to Referee 1

1) “more detailed summaries of the transcriptome and ChIPseq results should be provided in excel spreadsheets in the supplemental portion of the manuscript. For the RNAseq data, the relative values for each of the genes whose expression is altered upon CHIR treatment in WT and mutant cells should be provided. The reader should be able to use these spreadsheets to easily acquire the whole set of 139 genes that are regulated in a TCF and beta-catenin manner (and know how many are activated or inhibited by CHIR). Same for the other classes of genes described. For the ChIPseq data in WT and TCF mutant cells, the position of the beta-catenin peaks should be indicated, along with the nearest 5' and 3' gene.

We now provide all the data requested as Supplementary Tables (new Supplementary Tables 1-3). These tables include the list of statistically significant differentially regulated genes in the various cell lines that we used in the RNA-seq experiments, both in unstimulated and stimulated conditions.

We paid particular attention to producing tables that follow the logic of our experiments (as outlined in Figures 3 and 5) (new Supplementary Tables 1 and 2). Concerning the ChIP-seq experiments, we have added the peak lists with full annotations (i.e. chromosomal position, nearest transcriptional start site) (new Supplementary Table 3). Please also note that the RNA-seq and ChIP-seq raw data have been deposited at the ArrayExpress database (accession numbers E-MTAB-7029 and E-MTAB-7028, respectively).

2) In regard to the ~3900 beta-catenin peaks referred to in the manuscript, are there any controls to determine whether they are truly due to beta-catenin, e.g., ChIPseq in the absence of CHIR or in a beta-catenin mutant? It seems relevant to question the cleanliness of the beta-catenin antibody, given the fact that there are 27 of 166 genes that are regulated by CHIR/beta-catenin independently of TCFs, yet only 67 of the ~2300 beta-catenin peaks appear to be independent of TCFs.

We have now performed an entirely new set of ChIP-seq experiments using two different antibodies against b-catenin and have also performed the immunoprecipitation in dBCat cells (i.e. b-catenin KO). The experiments done with different antibodies show high genome-wide correlation (Supplementary Figure 6a). We are extremely grateful for having received this suggestion. Indeed, the new controls allowed us to exclude a significant number of potential ‘false positive’ signals and, most importantly, to focus our downstream analyses on those target regions that show the most stringent reproducibility (see new Figure 4, and Supplementary Figure 6a-c). Additionally, by intersecting the RNA-seq and ChIP-seq data, we now show that upregulated genes in WT are significantly more likely to be bound by β -catenin in WT than expected by chance (3.987 fold increase), providing additional validation of the specificity of the immunoprecipitation (Supplementary Figure 6d). Moreover, in d4TCF cells, b-catenin-dependent but TCF-independent genes become the group with highest association with ChIP-seq peaks (52.236 fold increase, Supplementary Figure 6f).

3) How many of the 166 CHIR regulated beta-catenin dependent genes in WT cells have a CHIPseq peak near them? Are any of the 67 TCF-independent CHIPseq peaks near genes that are regulated by beta-catenin but not TCF? Same for the 134 "ghost" targets and the 1600 new CHIPseq peaks. The authors should provide this data in table or figure form in the main body of the manuscript.

As briefly described in response to the reviewer’s previous point, the intersection between RNA-seq and ChIP-seq data indicated that b-catenin-dependent genes in WT cells are significantly more likely to be bound by β -catenin than expected by chance (3.987 fold increase, Supplementary Figure 6d). We defined as “bound gene” when a ChIP peak is found within 50kb from its TSS. This is the average distance of genes from CTCF peaks (~48kb), which indicates that anything outside this range is not likely to act as a cis-regulator. Interestingly, while genes upregulated in d4TCF cells are not significantly bound based on this parameter (Supplementary Figure 6d), the fraction of downregulated genes in d4TCF is more likely to be bound by b-catenin upon CHIR stimulation (d4TCF stim 9.136 fold, WT stim 9.489 fold, Supplementary Figure 4e). Finally, in d4TCF, the group of b-catenin-dependent but TCF-independent targets is the one with highest association with ChIP-seq peaks (52.236 fold increase, Supplementary Figure 6f). Together, these findings suggests a strict simultaneous requirement for TCF/LEF and b-catenin in WT cells, whereas b-catenin is relocated to TCF-independent target genes in d4TCF cells. We include the new analyses in the revised Figure 4 and Supplementary Figure 6, and describe the results in the main body of the manuscript.

4) Another key question that the authors' data should address is the percentage of Wnt targets in HEK293T cells that are subject to TCF repression in the absence of CHIR, i.e., are these genes expressed at higher levels in TCF mutants compared to WT?

We thank Reviewer #1 for this valuable observation. We have now analysed our dataset to precisely address this point. In the revised Figures 2c and 2c' we now show that, when compared to unstimulated WT parental cells, d4TCF cells displayed broader gene expression changes than dBCat cells in the absence of CHIR treatment. More specifically, in dBCat cells 120 genes are differentially expressed (50 up, 71 down) compared to WT, while in d4TCF this number raises 7-fold to a surprising 892 (391 up, 501 down). We interpret this difference as an indication of the important role that TCF/LEFs play in basal conditions, by binding on the WRE on the DNA even in the absence of active Wnt signalling.

Surprisingly however, we did not observe canonical target genes being “de-repressed” (i.e., upregulated in d4TCF cells). We believe that this is due to the fact that these analyses have been performed long after the “acute” induction of mutations in TCF/LEF-encoding genes (that could momentarily lead to “de-repression” of target genes), and account for many expected secondary and tertiary effects on gene expression caused by the genetic removal of the four transcription factors.

5) I have several issues with the way the manuscript is currently written:
a) I really don't like the title. What about something a little less dramatic, e.g., TCF dependent and independent regulation of Wnt/beta-catenin transcription.

We agree that a “less dramatic” title would better reflect our findings by providing a more balanced view of the TCF-dependent and independent roles of b-catenin. We changed the title as suggested to “TCF/LEF dependent and independent regulation of Wnt/b-catenin transcription”. Considering also the comment from Referee 2, we decided to remove the mention to the b-catenin “GHOST” activity from the title.

b) I strongly object to the statement that “the central tenet” of the Wnt pathway is exclusive regulation of targets by TCFs. This statement needs to be toned down. Many, many Wnt researchers are open to the possibility that some Wnt targets are regulated by non-TCFs.

We have toned down this statement, both in the abstract and in the introduction. We now refer to this by suggesting that “the activity of nuclear b-catenin is largely mediated by the TCF/LEF” and “The activation of target genes by the β -catenin/TCF complex has been established as the main modus operandi of canonical Wnt signalling”.

c) page 3: I don't like when researchers exclude GSK3a from the Wnt pathway. CHIR inhibits both GSK3a and GSK3b, and both kinases need to be mutated to see strong Wnt gain of function phenotypes in mammals. Just use GSK3 and you can help to correct the wide misconception that GSK3b is the more important kinase, or direct me to data that support that claim, other than the fact that everyone repeats the same mistake.

We agree with the Reviewer and apologise for overlooking the potential role of GSK3a. We have now specified in the text that when we refer to GSK3 we mean both kinases (page 3).

d) page 6: the first paragraph oversimplifies the debate over TCF/non-TCF mediation of Wnt gene regulation in the literature. Provide a more balanced view.

We have modified the text to provide a more comprehensive and balanced view of TCF/non-TCF mediation of Wnt gene regulation including several instances describing the action of b-catenin with non-TCF alternative transcription factors.

e) page 7: Axin2 is “a” prototypical target, not “the”

We agree. Done.

f) page 8: change “In our set up” to “Under our conditions”

Changed.

g) page 9, line 2: I don't like the use of the term “direct” when “beta-catenin dependent” would suffice. Direct implies a direct biochemical connection in the view of many researchers.

We agree and have removed the term “direct” as suggested.

Response to Referee 2

General. I really do not like the title and naming of this phenomenon as Ghost... It does not add anything just confusion. B-catenin GHOST makes me think of a real/(i.e.)identified GHOST TF with that name, that of course is different from the enigmatic GHOST(s?) phenomenon by which b-catenin operates as the authors are hinting. As external example, the Wnt-STOP signaling is different because it is an acronym (STabilization Of Protein). Just call this differently: TCF/LEF independent transcription or similar statements would suffice.

As both reviewer 1 and reviewer 2 suggested a similar problem with the original title, we changed the title of the revised manuscript to the more moderate “TCF/LEF dependent and independent regulation of Wnt/b-catenin transcription”. We also decided to remove the reference to “GHOST” activity from the title. This, we believe, provides a more balanced view on the different roles of b-catenin.

In Figure 3 we describe a set of the b-catenin-dependent genes whose regulation occurs both in the presence and absence of TCF/LEF proteins, and refer to these as “TCF-independent”. In the data displayed in Figure 5, we observed that a set of b-catenin-dependent genes respond to b-catenin activity *only in the absence* of TCF/LEF transcription factors, or when the TCF-b-catenin interaction is inhibited. In other words, they are not regulated in parental control cells, but depend on activated b-catenin in d4TCF cells. This set of genes (now additionally validated by RNA-seq) are not only TCF-independent, but require, for their transcriptional regulation, that TCF/LEFs are absent (or the TCF-b-catenin interaction inhibited). For this reason, we believe it is important to distinguish this group of genes from the “simply” TCF-independent targets and refer to it as GHOST (the acronym of genes hidden outside the standard targets) response.

1) A key missing experiment is the use of clones of the TCF/LEF CRISPR generated clones (d4TCF) reconstituted with at least 1 of these factors.

There is currently no rescue experiment. The main problem here is very technical: they are subcloning and subcloning and subcloning their 293 cells in order to make such 4x KO reagent

The Reviewer refers to a potential “bottleneck effect” caused by the single cell-based clonal selection performed from the different “Crispered” cell populations. We recognize the plausibility of such an effect and, in the original manuscript at page 8, we described that “we generated three independent clonal cell lines for each genotype to exclude the possibility of a bottleneck effect (i.e. the generation of clonal cell populations, via single cells, might affect the overall behavior of each clone)”. However, we agree with the reviewer that the rescue experiment is an important piece of evidence that was missing.

We performed a rescue experiment using two independent d4TCF (quadruple TCF/LEF KO) cell clones and show the results in Figure 1e, f and Supplementary Figure 1b, c. In a luciferase Top-flash assay, TCF7, LEF1 and TCF7L2 can reconstitute to different extents the transcriptional activity of the reporter when cells are stimulated with Wnt3a, while in the same experiment, both non-transfected d4TCF clones fail in activating the reporter (Figure 1e and Supplementary 1b). Similar results are obtained when CHIR is used instead of Wnt3a, and *Axin2* mRNA is measured (Figure 1f and Supplementary 1c). This indicates that d4TCF cells lost their ability to respond to Wnt stimulation only due to the absence of TCF/LEF factors, rather than alternative genetic/genomic hits as a consequence of clonal selection. It is interesting to note that TCF7L1 is the only factor that fails in reactivating the reporter transcription. This is consistent with previous work from Merrill’s group (e.g. Yi et al., 2013) and others, revealing an antagonistic relationship between canonical Wnt and TCF7L1 on gene expression. We interpret this observation as additional evidence that the clonal HEK cell lines we generated display a quasi-physiological response to Wnt pathway stimulation.

2) The second main problem for me is in the numbers. Upon CHIR-GSK3 inhibition (as proxy of Wnt stimulation), B-catenin controls 231 genes (81 Up and 150 Downregulated). In absence of TCFs, only 4 genes are up (and upregulation by nuclear b-catenin is by far the most understood phenomenon). 23 genes are downregulated without TCF. What to make of all those repressed genes is unclear, as this is hard to rationalize. it can be a direct effect or an indirect effect caused by the lack of a positive target of b-catenin. This is to say that there are many reasons for changing gene expression. And, irrespectively, 4 genes out of 81 is a mere 5%. Are these genes at least directly binding b-catenin in their cis-regulatory promoter or enhancer elements?

We were also surprised to observe that a consistent fraction of the differentially expressed genes upon CHIR treatment were downregulated. Note that these data, however, have been obtained at the time-point when CHIR induces the highest expression of *Axin2*, which is 24h. We did not imply, in our study, an expectation to identify direct b-catenin targets (i.e. whose regulation relies on direct biochemical interaction between b-catenin and their regulatory regions) at this time point. We acknowledge that the analysis therefore might include secondary or tertiary effects on gene expression that are ultimately executed by mechanisms other than direct positive regulation by the TCF/b-catenin complex. We wish to point out, in addition, that 231 genes display an altered transcriptional profile upon GSK3 inhibition – and many of them likely as a consequence of the

action of non-b-catenin related mechanisms (e.g. WntSTOP). Among these 231, in our experimental setup, 166 are b-catenin-dependent [72 of which are positively induced by b-catenin (*Axin2*, *Lef1*, *Sp5* and other canonical targets) and 94 are downregulated downstream of b-catenin action (this information is now included in new Supplementary Tables 1-2)].

We obtained these results in a triplicate RNA-seq experiment, and many of these gene expression changes have been confirmed by RT-qPCR in independently generated cellular clones. As such, we consider this a very robust dataset.

27 out of 166 b-catenin-dependent gene expression changes appear to be TCF-independent. This represents a considerable fraction accounting for more than 10% of the total gene expression changes, whose regulation occurs by definition via different mechanisms than the classical TCF/b-catenin association. It is interesting to note that our new analyses (Supplementary Figure 6) indicate that in WT cells, upregulated genes are significantly more likely to be bound by β -catenin than expected by chance (3.987 fold increase), whereas downregulated genes in d4TCF are more likely bound by b-catenin (d4TCF stim 9.136 fold, Supplementary Figure 4e). At this stage, we can only speculate that b-catenin, independently from TCF/LEF might interact with other transcription factors, some of which may well be transcriptional repressors – thereby explaining the high proportion of downregulated genes among the TCF-independent b-catenin-bound targets. We feel that investigating this is beyond the scope of the current work, but it does represent a very interesting follow up line of investigation.

Again the problem behind my questioning is certainly not raising silly doubts on undefined alternative hypotheses but it is quite cogent to the point at stake: are we truly observing a b-catenin induced transcription independent of TCF, or, rather, an indirect effect caused by whatever functions b-catenin may have in the cytoplasm? or due to the fact that b-catenin is anyway part of the destruction complex (that work on several other proteins beyond b-catenin in Wnt-off conditions, see Wnt STOP and other related stories), and thus in presence of CHIR+b-catenin KD still many proteins may get stabilized leading to some changes in gene expression down the road.

We appreciated the suggestion of these alternative explanations. At this stage, we could only conclude that, in the absence of b-catenin, some TCF-independent targets lose their CHIR-induced regulation (therefore, we define them as dependent on b-catenin presence). It remains plausible that the presence of b-catenin within the destruction complex can influence their regulation. We now acknowledge this possibility in the discussion section (page 15) of the revised manuscript. It is true that we present no evidence indicating that they are direct b-catenin transcriptional targets, but we also observed that while upregulated genes are likely bound by b-catenin in WT cells, b-catenin peaks are more likely found in the proximity of downregulated gene in a TCF/LEF KO context. At this stage, we believe that this might suggest a direct action in cooperation with transcriptional repressors.

Please also note (page 11) that 40% of genes modulated by CHIR are neither TCF nor b-catenin dependent, which is very interesting but just to say that there can be a broad array of fluctuations (and technical/biological intersection thereof) that may explain 5% of differential gene-expression changes.

We acknowledge the existence of a large fraction of genes modulated by CHIR but being independent of both TCF/LEF and b-catenin, and we interpret them as the potential consequence on the proteome of GSK3 inhibition (via mechanisms such as the WntSTOP). We describe this in our manuscript (page 9). We believe that we have constructed a robust strategy to identify expression changes that strictly depend on b-catenin (by generating b-catenin KO cells) on TCF/LEF (with the quadruple TCF/LEF KO cells) or on b-catenin in the absence of TCF/LEF (quintuple b-catenin and TCF/LEF KO cells), and to avoid experimental fluctuation by performing in-depth RNA sequencing in triplicates (Figure 2, Supplementary Figure 1), validating many targets via RT-qPCR (Figures 1-3 and 5), and generating independent cellular clones for each of the mutated cell line (Supplementary Figures 1 and 4).

3) Connected to the above, of course I also appreciate (page 10) that, moving from gene expression to genome occupancy, they also find "1600 statistically significant new enriched regions (i.e. occupied by -catenin) in d4CTF cells (Figure 5a). " This gives me confidence that what they are describing may be real. Only that I find the story still preliminary at this stage, requiring some reinforcements and better controls.

We have now performed an entirely new set of ChIP-seq experiments (revised Figure 4 and Supplementary Figure 6). Based also on the suggestion given by another referee, we performed the immunoprecipitation in dBeaT cells (i.e. b-catenin KO). Additionally, we made use of two different antibodies against b-catenin to further strengthen the dataset. These experiments show high genome-wide correlation among them (Supplementary Figure 6a). The new controls, importantly, allowed us to exclude a significant number of potential ‘false positive’ signals and to focus our downstream analyses on those target regions that show the most stringent reproducibility (Supplementary Figure 6b-f). We have noted however that different ChIP-seq display different efficiencies, which ultimately leads to differential peak-calling (Supplementary Figure 6b). In other words, the more efficient experiments result in higher numbers of reads-enriched regions that “surpass” the statistical threshold to be named “peak”. Thus, in the revision of our study, we decided to focus on those target regions that display very high correlation and reproducibility across replicates. We understand this might lead to the loss of potentially interesting information (i.e. false negatives), but we are now more confident that the smaller set of targets we present are ‘robust’ biological events. We present the data deriving from three independently generated ChIP-seq datasets – including both canonical and TCF-independent peaks – as well an entirely new bioinformatics analyses, in the revised Figure 4 and Supplementary Figure 6.

What about the FOXO connection:

a) is B-catenin binding to FOXO?

The interaction between b-catenin and FOXO factors - among which FOXO4 - have been previously shown in co-immunoprecipitation assays (Essers et al., 2005; Hoogeboom et al., 2008). We now provide additional evidence indicating that b-catenin and FOXO4 also physically associate with each other in our experimental system, and that their association appears to be dose-dependent (Figure 5c).

b) Can they try chip-rechip with b-catenin and foxo antibodies?

We agree that this would provide powerful evidence for a co-regulation of target genes by b-catenin and FOXO4. Unfortunately, we failed in performing ChIP-seq experiments using FOXO4 antibodies in several trial experiments. This indicated that the even more ambitious ChIP-reChIP approach was not feasible. However, we wish to point out that we strengthen the evidence of a b-catenin/FOXO4 cooperation, by (i) showing that they physically interact (revised Figure 5c), by (ii) finding that all the TCF-independent but b-catenin-dependent peaks display a FOXO binding motif (Figure 4e,f), and (iii) by combining overexpression with downregulation experiments of *FOXO4* (revised Figure 5f and Supplementary Figure 9). Note that the new experiment, in which we performed siRNA-mediated knock-down of *FOXO4*, strongly suggests that several of the TCF-independent but b-catenin-dependent targets are sensitive to *FOXO4* downregulation (that is, they require FOXO4 for their b-catenin-dependent regulation, Figure 5f and Supplementary Figure 9).

4) Are these FOXO sites within or adjoining the TCF motifs? and for how many loci? if not, How to explain the fact that this FOXO -responses and in general this TCF-independent phenomenon is, in truth, only detected in absence of TCF (and not just irrespectively of)

We performed new Motif search analyses including the new ChIP-seq experiments we have performed. It is worth noting that when we focus on the smaller subset of highly robust and reproducible targets, we identified the FOXO consensus binding sequence as the highest enriched motif (Figure 4e). While the peaks in WT cells show a higher fraction of TCF motifs, as expected, all b-catenin peaks in d4TCF display FOXO motifs (Figure 4e, f). In other words, all peaks with TCF motifs in d4TCF also have a FOXO motif, and together with the evidence we provide for the interplay between b-catenin and FOXO4, this could explain how ChIP enrichment is retained in several regions in d4TCF cells. These binding events are by definition TCF-independent, in that they occur in cells devoid of any TCF/LEF protein. The enrichment of the FOXO motif only in d4TCF cells could be explained by the loss of the majority of the remaining peaks that depends on TCF/LEF presence only, and that are associated with statistical recurrence of the TCF/LEF consensus alone (see Figure 4f).

5) an interesting finding is where they describe that b-catenin is still bound to TCF sites in absence of tcf lef proteins. Fig4. They write that “curiously, motif analysis of these 67 peaks shows a significant presence of TCF4 and TCF3 consensus binding sequences. This is all based on motif finder predictions, which is OK but do they know, by ChipSeq or ChipPCR, if all or a group of these genes is in fact really bound in the control parental cells by TCFs? If so, this would make a stronger

case in favor of another set of TFs able to bind and recruit b-catenin that are not TCFs, but may perhaps compete with TCF in normal conditions, new forms of default repressions etc. and other interesting ideas (to be discussed, perhaps).

We have now analysed the binding pattern of b-catenin in respect to TCF4, by comparing our dataset with the ChIP-seq data previously generated by the ENCODE Consortium (see Figure 4a,b and c). Many canonical peaks we identified were previously shown, expectedly, to be bound also by TCF4 in HEK 293T cells. It is interesting to note that the genome-wide signal of β -catenin is lost over TCF7L2 (TCF4) peaks in both d4TCF and dBcat cells. However, it is also interesting to observe that several of the TCF-independent b-catenin peaks displayed TCF4 occupancy (see the line above, in the genome browser views of Figure 4a and b). We emphasize this observation in the discussion section, when we state on page 15 that: “It is possible that this second binding behaviour reflects the fact that b-catenin relies on the presence of TCF/LEF in cooperation with other transcription factors, such as FOXO4. In this scenario, the removal of TCF/LEF could attenuate, but not fully abolish, b-catenin occupancy at this region. This interpretation is further supported by the high presence of proximal TCF and FOXO binding motifs within peaks representing TCF-independent binding events (Figure 4)”.

Response to Referee 3

1. GSK3 is involved in many cellular processes and does not act exclusively in the canonical Wnt pathway. Therefore, it is not clear whether authors measure genuine Wnt pathway responses upon treatment with CHIR. Stimulation with Wnt growth factors should have been performed.

The reason why we decided to use CHIR instead of Wnt3a is that, in a series of pilot experiments, only CHIR could sustain a robust and reproducible set of differentially expressed genes, which included several known Wnt/b-catenin canonical targets. We wish to point out that Wnt pathway stimulation in cultured cells is a challenge that many researchers in the field are dealing with; as a result of their acylation, Wnt proteins are highly hydrophobic and less prone to be purified and used in experimental applications than other protein ligands (see for example Janda CY et al. 2017). In addition, it is likely that synergistic activity between different Wnt ligands (Alok et al., 2017, from the Virshup group) or between Wnt and R-spondin (see for example Yan et al., 2017, from the Kuo lab) is required for full Wnt pathway activation. This is currently a major area of research which is beyond the scope of the current study. Importantly, the strategy we present here is precisely designed to deal with the potential “pleiotropic” effect of GSK3 inhibition. In particular, we could define the set of b-catenin-dependent gene expression changes - within the broader CHIR-mediated response - by comparing the output, on a transcriptome-wide level, between control parental cells and b-cateninKO cells (Figure 2, see also the new Figure 2c). This allowed us to establish a consistent b-catenin-dependent signature which does, very likely, exclude other effects mediated by GSK3 activity, such as the WntSTOP response.

2. Although widely used as a test tube to study Wnt signalling mechanisms, it is not clear whether HEK293 cells can activate a physiologically relevant gene expression program in response to Wnt pathway activation. Likewise, it is completely unclear whether HEK293 cells are equipped to trigger meaningful TCF/LEF-independent gene expression. For this, relevant transcription factors would have to be expressed at sufficient levels. In view of this, the observations made could be an artefact of the HEK293 cell system and the importance of the conclusions is questionable. In support of this concern, the number of genes whose expression changes under the various experimental conditions seems rather low. The principle component analysis and the hierarchical clustering further suggest that differences among the cells arise from genotype not treatment.

We agree about the limitations of the cellular system we used. However, we wish to point out that the strategy we selected (the generation of multiple CRISPR/Cas9-mediated multiple mutations in a single cellular clone) would be unfeasible in cultured primary cells. Among the various cell lines we decided to study this phenomenon in HEK 293T, for they have been widely used to investigate the biochemical events underlying the Wnt signalling pathway (see for example elegant work from the Clevers' group, Li et al., Cell, 2012 and Schuijers et al., EMBO Journal, 2014). Moreover, unlike other cell lines used to study Wnt signalling, such as the colon cancer derived SW480 or HCT116, HEK 293 do not carry pathway-specific activating mutations (Gujral & MacBeath, 2010), and are thus an appropriate *in vitro* model to investigate the differences between pathway “on” and “off” conditions. Finally, we have shown by several means that HEK 293T are highly responsive to Wnt-pathway activation (Figure 1c, d; Figure 2a Figure 4; Supplementary Figure 2, 3 and 8). Note, for example in Figure 2a, that among the “top” upregulated genes upon GSK3 inhibition there are several “paradigmatic” Wnt target genes (e.g. *AXIN2*, *DKK1*, *NKD1*, *SP5*), or that b-catenin binds to

previously defined WRE on the DNA only in the presence of TCF/LEF factors (Figure 4). We also now provide new data (see also comments from Referee 2) showing that different independently generated clones respond in a similar fashion (both quantitatively and qualitatively) to GSK3 inhibition, and their ability to activate the pathway is rescued when at least one among TCF7, LEF1 and TCF7L2 expressing plasmids is transfected (revised Figure 1e, f). Importantly, in this rescue experiment, TCF7L1 is the only factor that fails in reactivating the reporter transcription, consistent with previous work from the Merrill's group (e.g. Yi et al., 2013) and others that revealed an antagonistic relationship between canonical Wnt and TCF7L1 on gene expression. Taken together, we believe that these data underlie the intrinsic robustness of HEK 293T cells in switching from a "Wnt off" to a "Wnt on" status.

3. The authors hypothesize that activation of the β -catenin-GHOST response relies on the interaction with low affinity transcription factor binding partners and becomes visible only in the absence of TCF/LEF proteins or when their interaction with β -catenin is prevented. There is no experimental evidence for this model. Again, the authors may be misled by the specific conditions in HEK293 cells. In fact, in physiologically relevant models, relative expression levels of TCF/LEF and non-TCF/LEF binding partners with similar affinities for β -catenin could even allow for co-existence of TCF/LEF-dependent and independent gene expression. Thus, there is no justification for invoking low affinity interaction and the need of TCF/LEF absence or inhibition.

We agree with this note. In the revised manuscript we no longer provide any explanation concerning "low-affinity" transcription factors.

4. The authors claim that 15 β -catenin-GHOST response genes were analysed but data are shown only for 9 genes, all of which are β -catenin-dependent. How do the authors arrive at a quote of 60% β -catenin-dependent genes?

In the original version of the manuscript we aimed at validating the β -catenin-GHOST targets via RT-qPCR. Out of 15 β -catenin-dependent genes that we tested, 9/15 (60%) ceased being regulated in the absence of β -catenin (thereby confirming being β -catenin dependent), while 6 were activated irrespective of β -catenin (these 6 were previously shown in the supplementary material). We now provide a new data set describing RNA-seq analysis of pentaKO cells (lacking all TCF/LEF factors and β -catenin). This allowed us to precisely identify 97 genes that respond in a β -catenin-dependent manner in the absence of TCF/LEF. It seems therefore that an even higher proportion (90/107, ca. 85%) of the previously identified β -catenin-dependent genes behave as "GHOST" genes (regulated by β -catenin only in a context devoid of TCF/LEF, or in the presence of TCF- β -catenin interaction inhibitors, such as ICAT). We describe the new data in the revised manuscript and in Figure 4, along with their validation by RT-qPCR (Figure 5b). Please also refer to our response to Point 5 (see below) for a more detailed explanation.

5. How can there be β -catenin-independent genes among this group which according to the scheme in Figure 5b by definition should be β -catenin-dependent?

Among the 196 genes regulated by CHIR in d4TCF, 134 are not regulated by CHIR in dBCat cells (Figure 5a). We worked on the assumption that genes deregulated by CHIR in WT or in d4TCF cells, but not in dBCat cells, could be β -catenin-dependent. Part of this set (27/134) consists of the previously identified group of β -catenin-dependent but TCF-independent targets (Figure 3a). The remaining genes (107/134) are CHIR-regulated only in d4TCF cells (and not in WT nor in dBCat cells). We decided to distinguish the group of β -catenin-dependent genes (27) whose regulation occurs both in the presence and absence of TCF/LEF proteins (referred to as "TCF-independent", Figure 3a), from that responding to β -catenin activity *only in the absence* of TCF/LEF transcription factors (to which we refer as "GHOST response"). However, there is a subtle but crucial aspect we wish to point out. The original definition of β -catenin-dependent targets was obtained by including CHIR-mediated changes observed in WT cells, that stopped being regulated by CHIR in dBCat cells. It remained possible that the 107 genes shown in Figure 5a, induced by CHIR in d4TCF cells but not in WT or dBCat cells, are not regulated by CHIR in dBCat *not* because of the absence of β -catenin, but because of the presence of TCF/LEF in dBCat cells (as we find that they can only be regulated by CHIR when TCF/LEF are removed or when inhibitors of the β -catenin/TCF interactions are administered). To exclude this possibility, we provided additional evidence for the existence of a β -catenin-GHOST response: we induced loss-of-function mutations in *CTNMB1* in d4TCF cells (that is, we removed β -catenin "on top of" all the 4 TCF/LEF proteins), generating penta KO (pentaKO) cells. We could therefore identify the gene expression changes reproducibly appearing in d4TCF cells but not in pentaKO cells, and defined this " β -catenin-GHOST response" - the ensemble of

those genes that are regulated in d4TCF cell (note: not in parental control cells) and cease being regulated by CHIR in pentaKO cells - thereby identifying b-catenin dependent targets in a TCF/LEF-KO context. The new RNA-seq experiment performed on CHIR-stimulated pentaKO cells allowed us to identify 90 genes (all belonging to the previous group of 107) that are up- or down-regulated in d4TCF cells but do not change or are undetected in pentaKO cells (revised Figure 5). In the revised manuscript we now include a better description of this section (page 12) and in the revised Figure 5a we specify with more clarity the meaning of the different groups we identified via differential gene expression analyses.

6. Is the 60% fraction representative for the entire β -catenin-GHOST response gene set? If so, the β -catenin-GHOST response gene set would be considerably smaller than 107. Accordingly, what is the true size of the β -catenin-GHOST response gene set, i. e. genes inducible by CHIR in dTCF4 cells and β -catenin-dependent?

We hope we have provided a comprehensive explanation to this point in our response to point 5, above. Please consider that we have now added a new RNA-seq experiment which allowed us to identify more precisely the number of b-catenin GHOST targets, instead of estimating them based on RT-qPCR validation.

7. How do the five genes analysed in Suppl. Figure 6 fit into the picture? Are they among the 107 potential β -catenin-GHOST response genes? The legend to Suppl. Figure 6 states that these genes are induced by CHIR in dTCF4 cells. Aside from the fact that the corresponding bars are hard to make out it appears to me that ARC2 and TVP23C do not show any kind of significant regulation. Again, are these false positives in the RNA-seq data set?

The genes shown in the original Supplementary Figure 6 were those displaying similar CHIR-dependent activation pattern in d4TCF and pentaKO (thus behaving irrespective of b-catenin. This was presented to show that not all of the genes identified behave as b-catenin “GHOST targets”. This graph has now been removed in favour of a more comprehensive RNA-seq experiment (Figure 5, Supplementary Tables, see also response to point 5).

8. The authors insinuate that the β -catenin-GHOST response genes are regulated by β -catenin in non-TCF transcription factor complexes. This model could easily be corroborated (or refuted) by intersecting the true β -catenin-GHOST response genes with the β -catenin ChIP-seq data. Are confirmed β -catenin-GHOST response genes associated with β -catenin ChIP-seq peaks?

We have performed an entirely new analysis which included the intersection between RNA-seq and ChIP-seq data sets. We found that b-catenin-dependent genes in WT cells are significantly more likely to be bound by β -catenin than expected by chance (3.987 fold increase, Supplementary Figure 6d). We defined as a “bound gene” when a ChIP peak is found within 50kb from its TSS. This is the average distance of genes from CTCF peaks (~48kb), suggesting that anything outside this range is unlikely to act as a cis-regulator. Interestingly, while genes upregulated in d4TCF cells are not significantly bound based on this parameter (Supplementary Figure 6d), the fraction of downregulated genes in d4TCF is more likely to be bound by b-catenin upon CHIR stimulation (d4TCF stim 9.136 fold, WT stim 9.489 fold, Supplementary Figure 4e). Finally, but most importantly, in d4TCF cells b-catenin-dependent but TCF-independent become the group with highest association with ChIP-seq peaks (52.236 fold increase, Supplementary Figure 6f). This suggests a strict simultaneous requirement for TCF/LEF and b-catenin in WT cells, and that b-catenin is relocated to TCF-independent target genes in d4TCF cells. We include all these new observations in the revised Figure 4 and Supplementary Figure 6.

9. As an extension of this, genome browser views of examples for newly acquired β -catenin ChIP-seq peaks in d4TCF cells need to be shown.

Please note that we have now performed and entirely new set of ChIP-seq experiments (revised Figure 4 and Supplementary Figure 6). Importantly, we performed the immunoprecipitation in dBcat cells (i.e. b-catenin KO) and with two different antibodies against b-catenin (these experiments show high genome-wide correlation among them, Supplementary Figure 6a).

We have noted however that different ChIP-seq display different efficiencies, which ultimately leads to differential peak-calling (Supplementary Figure 6b). In other words, the more efficient experiments result in higher numbers of reads-enriched regions that “surpass” the statistical threshold to be named “peak”. The new experiment and the additional controls we have included, however, allowed us to exclude a significant number of potential ‘false positives’. We preferred therefore to focus our downstream analyses on those target regions that show the most stringent

reproducibility (Supplementary Figure 6b-f), thereby excluding several previously identified peaks that, despite their interest, were not significantly enriched in other experiments. For this reason, in the revision of our study, we decided to focus only on those target regions that display very high correlation and reproducibility. We realize that this might lead to the loss of potentially interesting information (i.e. false negatives), but we are now more confident that the smaller set of targets we present are ‘real’ biological events. We present the data deriving from three independently generated ChIP-seq datasets – including genome-browser view of both canonical and TCF-independent peaks – as well as an entirely new bioinformatics analyses, in the revised Figure 4 and Supplementary Figure 6.

10. The manuscript does not contain sufficient information about ChIP-seq peak location and differential gene expression under the different experimental conditions. The authors need to include supplementary tables listing ChIP-seq peaks and differentially expressed genes. This is especially important in relation to Figure 5b. A complete list of the 196 CHIR-responsive genes in d4TCF cells, the 27 CHIR-regulated but TCF-independent genes, and the β -catenin-GHOST response genes must be provided. In addition, RNA-seq and ChIP-seq data-sets should be made available to the scientific community by deposition in public repositories.

As outlined in response to Referee 1, we now provide all these data as Supplementary Tables (new Supplementary Tables 1-3). These tables include the lists of the differentially regulated genes in the various cell lines (RNA-seq), and the lists of candidate binding regions with full annotations (i.e. chromosomal position, indication of the nearest transcriptional start site, ChIP-seq). Please also note that the RNA-seq and ChIP-seq raw data have been deposited at the ArrayExpress database (accession numbers E-MTAB-7029 and E-MTAB-7028, respectively).

11. The authors present FOXO4 as an already known interaction partner of β -catenin that might contribute to the β -catenin-GHOST response. To make this point stronger and more convincing the authors should provide evidence that endogenous FOXO4 is expressed in HEK293 cells. On top of that it needs to be examined whether FOXO4 loss-of-function indeed affects the β -catenin-GHOST response.

FOXO4 is expressed in our HEK 293T cells – we know this primarily from the RNA-seq experiments (its expression does not change with CHIR treatment but the presence of its transcript is clearly detectable). Moreover, western blot analysis performed in the context of a new co-immunoprecipitation assay to test if FOXO4 interacts with β -catenin, definitively assesses the presence of endogenous FOXO4 protein (new Figure 5c).

Concerning the suggestion of FOXO4 loss-of-function experiments: we feel grateful for it. In the revised Figure 5f we show that siRNA-mediated FOXO4 downregulation does affect the expression of the β -catenin GHOST target genes (e.g. *GADD45*). More specifically, while *GADD45* transcription is induced in d4TCF cells in a β -catenin-dependent fashion upon CHIR treatment (Figure 5b), specific FOXO4 targeting siRNA – but not control scrambled siRNA – blocked *GADD45* induction (Figure 5f). This new evidence suggests that the expression of some β -catenin-GHOST targets (e.g. *GADD45*) relies on the activity of FOXO4. Please note that this new set of experiments is complementary to the overexpression (OE) studies we previously performed (Figure 5e) showing that FOXO4 OE can induce *GADD45* transcription in WT but not in d β cat cells, thereby emphasizing the requirement of β -catenin for its induction.

Minor issues:

We have modified/corrected all the following minor issues pointed out by Referee 3.

12. Figure 1: Please add Mw standard sizes to panel Fig. 1b; make spelling d β cat / d β cat consistent in panels Fig. 1b and c.

We added indication of the Mw sizes and corrected “d β cat”.

13. Figures 1d; 2e; 3b,c; 4c,d; Suppl. Figures 2, 3, 5, 6, 7: please add tick marks to y-axes.

Tick marks are now visible in all y-axes.

14. Figure 2e, Suppl. Figure 5: it would be more informative and allow for better comparison of genotype-dependent gene expression changes if the authors consistently applied the same experimental setup as used in Figure 1 d, i. e. measure FUT1 and HMOX1 also in WT/d β cat and in WT/d4TCF cells, respectively.

We have modified the two figures to make them consistent with the others.

15. Figure 3: To allow for full assessment of β -catenin and TCF/LEF dependent/independent gene expression changes please include dBCat cells in the analyses of CHAC1, SNHG7, ADAMTS18, TERC, etc.):

We included dBCat cell in the new gene expression analysis. It is shown in revised Supplementary Figure 6.

16. Figure 4: Please correct the fatal spelling errors Tef3 > Tcf3; Tef4 > Tcf4; Het116 > Hct116.

We corrected the spelling errors – thank you for identifying them.

17. Page 10: The authors mention that the 67 peaks which are occupied by β -catenin independently from TCF/LEF proteins show enrichment of binding motifs for HOXC/HOXD and MYB proteins and refer the reader to Figure 4d. However, this information is not included in the figure and consequently needs to be added.

Note that our new ChIP-seq analyses, which included additional controls, allowed us to refine our focus on a smaller but highly reproducible set of TCF-independent targets. It is interesting to note that, despite the increased stringency of the new analyses, motif search identifies the FOXO consensus as the only statistically significant motif in this group. We interpret this new result as a powerful validation of our downstream analyses shown in Figure 5.

17. Figure 5b: The Venn diagram does not properly reflect the numbers of genes whose regulation is specific and common to the two genotypes and needs to be corrected.

We now generated proportional Venn diagrams.

18. Suppl. Figure 6: The x-axis labels are incomplete; they do not match the numbers of bars and do not allow to identify treatment conditions.

For the reasons given in response to points 5 and 6, Supplementary Figure 6 has now been removed from the manuscript.

2nd Editorial Decision

3rd Aug 2018

Thank you for submitting the revised version of your manuscript. We have now received three referee reports, which are included below. Referees 1 and 2 are satisfied with the revised version, while referee #3 has some remaining concerns that need to be addressed. I have discussed these issues further with the referees and my colleagues. Below please find my comments on the issues and how to address them.

1. Referee comment: "As in the original version of their manuscript the authors used genome-editing, ChIP-seq and RNA-seq to arrive at the conclusion that there are TCF-dependent and TCF-independent gene regulatory events mediated by beta-Catenin (not a novel finding), that beta-Catenin can team up with non-TCF transcription factors (not a novel finding) and that some transcriptional responses become apparent only in the complete absence of TCFs (dubious significance)."

→ I see the point the referee is making, but also find the study provides important novel insight.

2. Referee comment: "This is because HEK293 cells simply do not provide a molecular milieu as would be found in the genuine expression domains of these genes. At best, distorted and crippled transcriptional responses will be elicited which do not measure up to what could be observed in vivo."

→ The other referees and I agree that HEK293 cells are still widely used for studying Wnt signaling, therefore there is no need for further experiments for this point.

3. Referee comment: "Use of GSK3 inhibitor as Wnt mimetic (original comment 1): The authors now use Wnt3a to quite successfully stimulate reporter gene activity (Figure 1e), demonstrating that pathway activation by a genuine Wnt ligand is possible. Thus, there is no reason for not using

Wnt3a at least for qRT-PCR validation of the genes examined in Figure 3b,c, Supplementary Figure 7, and especially of the GHOST-response genes in Figure 5. If, however, as claimed by the authors, Wnt3a cannot sustain a robust and reproducible transcriptional response in HEK293 cells, maybe this cellular model indeed is not suitable for this type of investigation casting serious doubts on the significance and relevance of the results.

→ GSK3 inhibitor is a standard method to induce Wnt signaling. However, we find that addressing this concern experimentally would strengthen the manuscript. The reason why Wnt3a does not sustain a long-term signal could be due to internalization of the Fz receptors. One of the referees suggested using Rspodin1 (to increase the Fz receptor amount on the cell surface) in combination with Wnt3a for qPCR validation of the GHOST response genes. This experiment should be fairly easy to do - let me know if we need to discuss this point further.

4. Referee comment: "Occupancy of GHOST response genes by beta-Catenin (original comment 8): For some reason the authors seem rather reluctant to answer this question in a straightforward way, even though with the newly added Supplementary Tables the comparison is quite simple: the authors identify 90 GHOST response genes (Suppl. Table 2) which by definition are TCF independent. However, in d4TCF cells there are only 24 beta-Catenin binding regions (Supplementary Table 3) that distribute across only 12 genes, none of which is part of the GHOST response set. In my opinion, this indicates either that the ChIP-seq data are not reliable, or that the GHOST response genes are regulated by beta-Catenin indirectly, or both. In any case, the data presented do not support the authors' model for how the GHOST response genes are regulated." (Also issue #1, 2 and 3 of the referee #3)

→ I discussed this issue further with the referees # 1 and 2. The reason why there is not a direct match between RNA-seq and ChIP-seq data could be a technical issue. Moreover as β -catenin does not directly bind DNA ChIP experiments and motif determination are not straightforward. Also, regulation does not necessarily occur at the cognate promoters if the genes, it could rather lay in the enhancer regions, which could be quite far away from the promoter region and the interaction could occur via DNA looping. An alternative explanation would be a regulatory CHIR dependent mechanism that is also β -catenin independent. I agree with the explanation of the referees, yet I believe discussing these issues in the manuscript is necessary.

5. Referee points #4, 5, 6

→ The referee points out the discrepancies that stands out in the presented data and suggests careful examination of them. Please address these comments.

6. Please address the additional issues raised by the referee #3 (points 8-12).

REFeree COMMENTS:

Referee #1:

I am fully satisfied with the revised manuscript. The additional controls and retooling of the text and data files make this an important paper for everyone interested in Wnt mediated gene regulation. The limitations of the cell type (everyone agrees that HEK293T cells are a laboratory creation) are strongly counterbalanced by the thoroughness of the analysis with CRISPR and ChIP-seq/RNAseq cross referencing. This comprehensiveness makes this report will be a must read for Wnt researchers.

Referee #2:

Fine for me. I am convinced by the revision

Referee #3:

The authors present a revised version of their study which aims to assess the relative importance of TCF/LEF proteins in Wnt/beta-Catenin-mediated transcriptional responses. During the revision the authors admittedly eliminated a great deal of my original criticism - albeit the less important aspects thereof - by making the necessary corrections, by performing new experiments, by adding new data, and by extended data analyses. Some criticism simply became obsolete in the process. Nonetheless, major concerns were not addressed, and several new issues became apparent upon disclosing data which had not been made accessible before. As in the original version of their manuscript the authors used genome-editing, ChIP-seq and RNA-seq to arrive at the conclusion that there are TCF-dependent and TCF-independent gene regulatory events mediated by beta-Catenin (not a novel finding), that beta-Catenin can team up with non-TCF transcription factors (not a novel finding) and that some transcriptional responses become apparent only in the complete absence of TCFs (dubious significance). Although the experiments were performed at a high technical level, the authors themselves admit in their manuscript that they chiefly confirm existing models for beta-Catenin mediated transcriptional regulation via its interaction with TCF family members. Likewise, it is already well known that beta-Catenin can cooperate with non-TCF transcription factors, including FOXO4. Thus, I do not see much novelty and conceptual advancement here. As to the GHOST response, this appears to be a negligible thing and there are serious inconsistencies in the data shown (see comments 3-7 below). A major weakness still is the choice of HEK293 cells as model system. It is my feeling that the authors overrate the significance of their findings because they study gene regulatory events in a cellular background which is inappropriate for many of the genes under investigation. This is because HEK293 cells simply do not provide a molecular milieu as would be found in the genuine expression domains of these genes. At best, distorted and crippled transcriptional responses will be elicited which do not measure up to what could be observed *in vivo*.

Instances where response to my previous criticism is not convincing/not satisfying:

Use of GSK3 inhibitor as Wnt mimetic (original comment 1):

The authors now use Wnt3a to quite successfully stimulate reporter gene activity (Figure 1e), demonstrating that pathway activation by a genuine Wnt ligand is possible. Thus, there is no reason for not using Wnt3a at least for qRT-PCR validation of the genes examined in Figure 3b,c, Supplementary Figure 7, and especially of the GHOST-response genes in Figure 5. If, however, as claimed by the authors, Wnt3a cannot sustain a robust and reproducible transcriptional response in HEK293 cells, maybe this cellular model indeed is not suitable for this type of investigation casting serious doubts on the significance and relevance of the results.

Use of HEK293 cells as model system in general (original comment 2):

I disagree with the authors concerning the feasibility of the work in other cells. For instance, there are the quadruple knock-out ES cells from the Doble lab. In addition, there are intestinal stem cell cultures which are amenable to CRISPR/Cas9 genome editing and offer all the other advantages of the HEK293 system. On top of that, both ES cells and intestinal stem cells would allow to study physiologically relevant regulation of gene expression upon Wnt pathway activation, something which definitely cannot be said for HEK293 cells. Of course, in intestinal stem cells the single knockout of TCF7L2 is lethal, demonstrating that the GHOST response, should it exist in these cells, probably is of no physiological relevance.

Occupancy of GHOST response genes by beta-Catenin (original comment 8):

For some reason the authors seem rather reluctant to answer this question in a straightforward way, even though with the newly added Supplementary Tables the comparison is quite simple: the authors identify 90 GHOST response genes (Suppl. Table 2) which by definition are TCF independent. However, in d4TCF cells there are only 24 beta-Catenin binding regions (Supplementary Table 3) that distribute across only 12 genes, none of which is part of the GHOST response set. In my opinion, this indicates either that the ChIP-seq data are not reliable, or that the GHOST response genes are regulated by beta-Catenin indirectly, or both. In any case, the data presented do not support the authors' model for how the GHOST response genes are regulated.

Novel issues arising with the revised version:

1. The authors claim that "beta-Catenin embarks on the regulation of different sets of genes and binds alternative genomic locations" in the absence of TCF proteins. In the introduction the authors

combine the two aspects and together describe them as GHOST response (page 5). I take it that the authors consider the GHOST response as their most significant finding. However, its importance shrinks considerably upon closer examination. beta-Catenin relocates to a mere 24 binding regions (compared to 1297 in WT cells) at 12 gene loci in the absence of TCFs, and none of these genes are regulated. Accordingly, TCF-independent gene regulation and beta-Catenin alternative genomic binding are different issues and need to be kept apart.

2. Figure 3b,c: The data show TCF independence but not beta-Catenin dependence. The authors' conclusions and statements on page 10 of the manuscript are not valid. In fact, beta-Catenin dependence of the genes in question is analyzed in Supplementary Figure 7 which shows regulation by CHIR of ADAMTS18, TERC, and EBF2 in dBcat cells, although the direction of regulation is reversed for ADAMTS18 and TERC. Nonetheless, half of the genes checked by qRT-PCR are also beta-Catenin independent. Furthermore, beta-Catenin dependence of a large fraction of the set of 27 genes is questionable based on Supplementary Table 1 where 17 out of 27 genes show statistically significant regulation in dBcat cells with the same directionality and often similar magnitude as in WT.

3. Figure 4b: HOXC4 and ZNF503 are presented as examples for supposedly TCF-independent beta-Catenin association. However, the genome browser views include a line showing TCF7L2 binding regions from the ENCODE data collection. These appear to be present also at the HOXC4 and ZNF503 loci where they coincide with beta-Catenin ChIP-seq peak regions. Importantly, beta-Catenin binding indeed does seem to be affected in d4TCF cells. In other words, TCF7L2 is present at so-called TCF-independent gene loci and contributes to beta-Catenin recruitment. Therefore, occupancy by beta-Catenin cannot strictly be called TCF-independent at the HOXC4 and ZNF503 loci. In view of this, how many robust and truly TCF-independent beta-Catenin binding events are there, which could represent the alternative genomic loci the authors refer to? Which are these loci? It seems there are merely gradual differences where the individual contributions of TCF and non-TCF transcription factors are subject to shifting weights with respect to their roles in recruitment of beta-Catenin at different cis-regulatory elements. All of this is perfectly compatible with models of combinatorial and multifactorial control of gene regulation but does not really provide novel and conceptually advanced insights.

4. Figure 4b: HOXC4 is not among the regulated genes listed in Supplementary Tables 1 and 2, including the GHOST response genes. What is the significance of the association of beta-Catenin with this locus? Are there more examples for non-productive beta-Catenin binding? Again, I would argue that observations like these question the relevance of the model system.

5. How do the authors explain that HOXC4 is not listed at all in Supplementary Table 3? How did they obtain the ChIP-seq tracks for beta-Catenin at this locus? Likewise, if ZNF503 represents TCF-independent beta-Catenin binding events why does it not appear in the d4TCF spread sheet in Supplementary Table 3?

6. Remarkably, ZNF503 and its antisense transcripts turn up as TCF-dependent genes in Supplementary Table 1! In agreement with this, ZNF503 and the antisense transcripts are not among the GHOST response group. Yet, the authors use this locus as an example for TCF-independent beta-Catenin occupancy. Can the authors explain this discrepancy?

7. Along the same line: While going over the data, the APCDD1 gene caught my attention. This is a fairly well known Wnt/beta-Catenin target gene. Consistent with this, APCDD1 shows up in Figure 2a with a nice CHIR response in WT cells, and it is listed in Supplementary Table 1 as a TCF-dependent gene. However, in Supplementary Table 2 APCDD1 appears among the 90 GHOST response genes. It is also listed as NOT CHIR-regulated in WT! This does not make any sense to me. I did not go over every single entry of the tables but I am getting a bit worried about data muddling. Therefore, I strongly recommend that the authors carefully check the data presented in their tables and figures for correctness, consistency, and plausibility.

Additional issues:

8. Figure 1e, Supplementary Figure 1b,c: The labeling of the x-axes is incomplete and confusing. The current versions suggest that cells alternatively received Wnt3a or the TCF/LEF rescue plasmid. It should be made clear that the TCF/LEF rescue samples were also treated with Wnt3a. In

Supplementary Figure 1c why did the LEF1 sample not receive CHIR?

9. Figure 2b,c: The legend does not match the panels. It was just carried over from the original version. It needs adaptation and proper description of the volcano plots.

10. Figure 4f, Supplementary Figure 6: Again, tick marks are missing from the y-axes.

11. Figure 5a: In contradiction to the authors' response to my previous request the Venn diagram has not been amended. It still suggests that 196 genes respond to CHIR solely in d4TCF cells which is in conflict with the manuscript text on page 11/12. The authors need to show the number of genes commonly regulated in WT and d4TCF cells in the intersection of the two circles and specify precisely how many of the 196 genes are exclusive to the d4TCF cells.

12. Figure 5f, Supplementary Figures 9 and 10: There is a problem with the labeling of the x-axis. The authors need to make clear that all samples represent experiments with d4TCF cells, i. e. add this information to the final two bars in Figures 5f and Supplementary Figure 9, and maybe replace d4TCF with "untreated" or "control" in Supplementary Figure 10.

2nd Revision - authors' response

29th Aug 2018

We have strived to address all reviewers' comments, with particular attention paid to correcting a series of inconsistencies identified by the thorough assessment of our work.

In particular, we have:

- Performed a new experiment by stimulating HEK293T cells with a combination of Wnt3a and R-spondin1 as a validation of TCF-independent target genes.
- Corrected inconsistencies identified by the scrutiny of Reviewer #3, and discussed these in the revised manuscript where appropriate.

We feel that these changes have further strengthened the clarity and conclusions of our article.

We appreciate the opportunity to submit our revised manuscript. Together with our co-authors we are looking forward to hearing from you.

DETAILED RESPONSE TO THE REVIEWERS

Below, we detail how we have addressed each point raised by the reviewers. Please note that referee's comments are copy-pasted in red, while our responses are in black.

Response to Reviewers #1 and #2

#1: I am fully satisfied with the revised manuscript. The additional controls and retooling of the text and data files make this an important paper for everyone interested in Wnt mediated gene regulation. The limitations of the cell type (everyone agrees that HEK293T cells are a laboratory creation) are strongly counterbalanced by the thoroughness of the analysis with CRISPR and ChIP-seq/RNAseq cross referencing. This comprehensiveness makes this report will be a must read for Wnt researchers

#2: Fine for me. I am convinced by the revision

We are grateful to Reviewers #1 & #2 for their careful and positive assessment of our work.

Response to Reviewer 3

During the revision the authors admittedly eliminated a great deal of my original criticism - albeit the less important aspects thereof - by making the necessary corrections, by performing new experiments, by adding new data, and by extended data analyses. Some criticism simply became obsolete in the process.

We are impressed by - and extremely grateful for - Reviewer #3's thorough assessment of our work. Below we detail, point-by-point, how we have addressed the additional criticisms and corrections raised by Reviewer #3.

Instances where response to my previous criticism is not convincing/not satisfying:

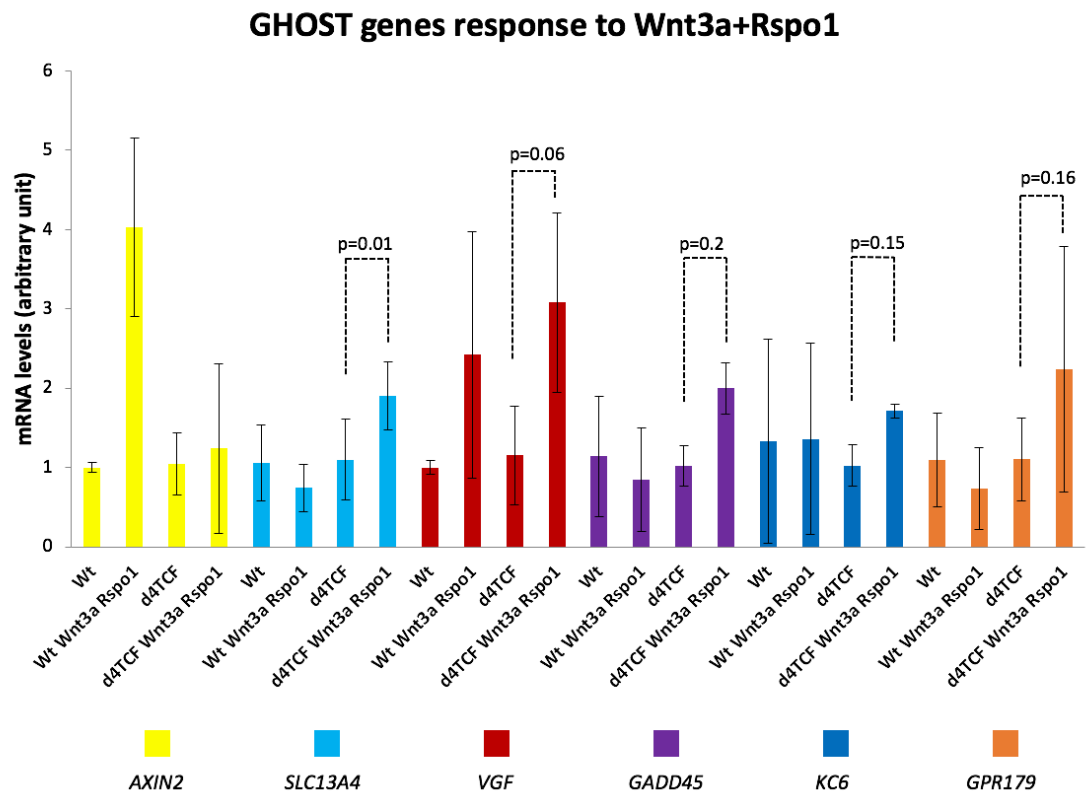
Use of GSK3 inhibitor as Wnt mimetic (original comment 1):

The authors now use Wnt3a to quite successfully stimulate reporter gene activity (Figure 1e), demonstrating that pathway activation by a genuine Wnt ligand is possible. Thus, there is no reason for not using Wnt3a at least for qRT-PCR validation of the genes examined in Figure 3b,c,

Supplementary Figure 7, and especially of the GHOST-response genes in Figure 5. If, however, as claimed by the authors, Wnt3a cannot sustain a robust and reproducible transcriptional response in HEK293 cells, maybe this cellular model indeed is not suitable for this type of investigation casting serious doubts on the significance and relevance of the results.

As we previously pointed out, Wnt pathway stimulation in cultured cells using individual Wnt molecules is a challenge that many researchers in the field are dealing with – regardless of the cellular model used. This is a current area of investigation, that is not, however, the focus of our study. For examples, as previously mentioned, see the generation of surrogate Wnt agonists by the Garcia group precisely aimed at addressing this issue (Janda CY et al., Nature, 2017), or work from the Virshup group, investigating the different responses mediated by different combinations of Wnt ligands (Alok et al., Journal of Cell Science, 2017).

A possible reason why Wnt3a does not sustain a long-term signal could be due to internalization of the Fz receptors. To test this, we performed a new experiment in which we stimulated HEK293T cells with a combination of Wnt3a and R-spondin1 (Yan et al., Nature, 2017) (Rspo1, to increase the Fz receptor amount on the cell surface), and measured via RT-qPCR the GHOST-response gene expression. We previously observed that, while Wnt3a was capable of inducing the activation of the artificial reporter TOPFLASH and *Axin2*, the response was limited to a small number of targets (RNA-seq, unpublished). By adding Wnt3a+Rspo1 in wild-type and d4TCF cells (see Figure below), we observed that a sample of “GHOST” genes were positively regulated, in agreement with our hypothesis (i.e. Wnt3a+Rspo1 tend to increase their transcription in d4TCF cells but not in wild-type).



While this experiment reinforces our confidence about the existence of a b-catenin dependent response in TCF/LEF-KO cells, and it is in agreement with the observations described in our study, the effect is modest when compared to that obtained by CHIR-stimulation, and the analysis lacks the statistical confidence (most of p-values are >0.05) for its inclusion in the manuscript.

We have discussed this point with the Editor, and agreed that administration of GSK3 inhibitors is an acceptable standard method to induce the Wnt pathway. This also considering that we adopted a strategy to exclude the effect of GSK3 inhibition that are not mediated by b-catenin (by isolating the transcriptional effects present in control parental cells but not in b-cateninKO cells, Figure 2).

Use of HEK293 cells as model system in general (original comment 2):

I disagree with the authors concerning the feasibility of the work in other cells. For instance, there are the quadruple knock-out ES cells from the Doble lab. In addition, there are intestinal stem cell

cultures which are amenable to CRISPR/Cas9 genome editing and offer all the other advantages of the HEK293 system. On top of that, both ES cells and intestinal stem cells would allow to study physiologically relevant regulation of gene expression upon Wnt pathway activation, something which definitely cannot be said for HEK293 cells. Of course, in intestinal stem cells the single knockout of TCF7L2 is lethal, demonstrating that the GHOST response, should it exist in these cells, probably is of no physiological relevance.

We have discussed the use of HEK293 cells with the Editor, and agreed that, despite their limitations (that we acknowledge in the manuscript), they are still a widely used model in the field, and there is no need of additional experiment to address this issue.

Occupancy of GHOST response genes by beta-Catenin (original comment 8):

For some reason the authors seem rather reluctant to answer this question in a straightforward way, even though with the newly added Supplementary Tables the comparison is quite simple: the authors identify 90 GHOST response genes (Suppl. Table 2) which by definition are TCF independent. However, in d4TCF cells there are only 24 beta-Catenin binding regions (Supplementary Table 3) that distribute across only 12 genes, none of which is part of the GHOST response set. In my opinion, this indicates either that the ChIP-seq data are not reliable, or that the GHOST response genes are regulated by beta-Catenin indirectly, or both. In any case, the data presented do not support the authors' model for how the GHOST response genes are regulated.

There are several reasons why a direct match between RNA-seq and ChIP-seq data might not be identified. First of all, technical reasons: β -catenin does not directly bind DNA, which makes ChIP experiments and motif determination challenging. Moreover, regulation does not necessarily occur at gene promoters, but it could depend on the interaction of β -catenin with distant regulatory elements. In this case, regulation is known to be mediated by DNA looping (Tae Hoon Kim et al., Cell, 2007; Chepelev et al., Cell Research, 2012). Another possible explanation could be a CHIR dependent regulatory mechanism that is also β -catenin-dependent.

We now discuss these possibilities more clearly in the main body of the manuscript:

- 1) On pages 10-11 we write: "In our analyses, we could not determine a direct relationship between TCF/LEF-independent peak-associated (ChIP-seq) and CHIR-regulated genes (RNA-seq). However, transcription factor binding does not necessarily occur at the proximal promoter of genes, but can take place at distant regulatory elements (Dickel et al, 2013). We therefore defined a gene as "associated" when a ChIP peak was found within 50 Kb from its transcriptional start site (TSS). This is the average distance of genes from CTCF peaks (~48kb) and interacting promoters (Kim et al, 2007)."
- 2) We explicitly present the difficulty in performing these experiments, and mention that different experiments resulted in different chromatin pull-down efficiencies: "While the number of TCF-independent b-catenin peaks appeared to be variable in different experiments, likely due to different pull-down efficiencies or perturbation of culture conditions (Supplementary Figure 6b, Supplementary Table 3), we identified a small subset of ca. 30 highly reproducible TCF-independent binding regions (Supplementary Figure 6c)." We now include in Supplementary Table 3 the results obtained in all 3 experiments, specifying for each experiment the pull-down efficiency, which we interpreted as a measure of the reliability of a given experiment.
- 3) We also acknowledge the possibility of an indirect action of b-catenin (i.e. non-mediated by physical association with the DNA) in the discussion section on page 16: "However, we cannot exclude the possibility, that TCF-independent b-catenin-targets reflect an indirect effect caused by cytoplasmic functions of b-catenin as part of the destruction complex in the absence of Wnt signals (Nusse & Clevers, 2017). The destruction complex, via the promiscuous activity of GSK3, acts on a plethora of proteins (Taelman et al, 2010), and it is possible that the genetic removal of b-catenin may affect the regulation of several genes as a consequence of its impaired activity."

Novel issues arising with the revised version:

1. The authors claim that "beta-Catenin embarks on the regulation of different sets of genes and binds alternative genomic locations" in the absence of TCF proteins. In the introduction the authors combine the two aspects and together describe them as GHOST response (page 5). I take it that the authors consider the GHOST response as their most significant finding. However, its importance shrinks considerably upon closer examination. beta-Catenin relocates to a mere 24 binding regions (compared to 1297 in WT cells) at 12 gene loci in the absence of TCFs, and none of these genes are regulated.

We thank Reviewer #3 for this important comment. Indeed, we greatly reduced the emphasis on the b-catenin GHOST activity in the previously revised version of the manuscript (for example, in the previous version we removed the mention to this phenomenon from the title), to reflect this point. Moreover, we wish to point out that, in studying the TCF-independent b-catenin occupancy, we chose a conservative approach by focusing on a small subset of highly reproducible TCF/LEF-independent peaks. We realize that, in this way, we might generate false negatives (i.e. the most efficient ChIP experiments identify real peaks that are however not reproduced in less efficient ones). For transparency, we include in the new Supplementary table 3 the individual results of all ChIP experiments. Also note that all the data have been deposited at the ArrayExpress database (accession number E-MTAB-7028).

Accordingly, TCF-independent gene regulation and beta-Catenin alternative genomic binding are different issues and need to be kept apart.

We clearly explain that TCF-independent gene regulation (described in Figure 3) and the b-catenin action in the absence of TCF/LEF (that we call GHOST-response, Figure 5), are two different phenomena. Accordingly, we clarify in the results section the potential “artificiality” of the GHOST response (page 12), that we attempted to address (successfully) by a more physiological inhibition of the TCF-b-catenin interaction (via ICAT overexpression, Figure 5).

2. Figure 3b,c: The data show TCF independence but not beta-Catenin dependence. The authors' conclusions and statements on page 10 of the manuscript are not valid. In fact, beta-Catenin dependence of the genes in question is analyzed in Supplementary Figure 7 which shows regulation by CHIR of ADAMTS18, TERC, and EBF2 in dBea cells, although the direction of regulation is reversed for ADAMTS18 and TERC. Nonetheless, half of the genes checked by qRT-PCR are also beta-Catenin independent.

We must agree that the former Supplementary Figure 7 was misleading, and in apparent contradiction with our statement on page 10. However, as we specified in the figure legend, the changes we obtained via RT-qPCR were statistically non-significant. We have now made this analysis more robust, by combining RT-qPCR validation from the samples used for RNA-seq with new experiments using CHIR treatment. The data obtained show that *CHAC1*, *SNHG7*, *ADAMTS18*, *TERC* genes do not change their transcription in the absence of b-catenin, thus indicating their dependence on this protein. One exception is *EBF2* (non-significantly downregulated, p-value=0.074). Importantly, our aim is not to draw conclusions on the behaviour of individual genes; our main message, on the other hand, is to emphasize the existence of TCF-independent but b-catenin dependent gene regulation on a transcriptome-wide scale.

Furthermore, beta-Catenin dependence of a large fraction of the set of 27 genes is questionable based on Supplementary Table 1 where 17 out of 27 genes show statistically significant regulation in dBea cells with the same directionality and often similar magnitude as in WT.

We identify a group of 27 genes that are significantly differentially expressed in CHIR-treated WT and d4TCF cells (ergo TCF/LEF-independent), but not significantly regulated in dBea cells (therefore, b-caterin dependent). This conclusion is based on the use of thresholds: specifically, we define a gene as significantly regulated when adjusted p-value < 0.05 AND absolute logFC > 1. The referee is pointing out that 17 genes (from the group of 27) appear transcriptionally regulated in dBea cells. However, they are excluded from the b-catenin-independent group precisely because their logFC was between -1 to 1 (therefore, their change was not statistically significant, according to our definition). We agree that there might be transcriptional fluctuations that complicate the attribution of individual genes in one group or in the other. However, these analyses must imply the setting of thresholds based on statistical parameters. We wish to point out that, by the same principle, it is very likely that we lose interesting candidates (false negative) precisely because they do not conform to the stringent cut-off we applied. Additionally, quite apart from drawing conclusion based on individual genes, the main message we wish to convey is the existence of such a mechanism (TCF-independent but b-catenin dependent gene regulation) and that this phenomenon is detectable with our approach.

Finally, but importantly, we validated handful of genes for each relevant group via RT-qPCR, also by using independently generated cellular clones (Fig. 2e, f; Fig. 3b, c; Fig. 5b; Supplementary Fig. 5, Supplementary Fig. 7), thereby confirming the overall reliability of our interpretation of the RNA-seq data.

In the revised manuscript we have altered the results section to more clearly describe how we defined a statistically significant change, and rephrased the paragraph as follows (pages 9-10): “We identified a set of 27 b-catenin-dependent genes that are regulated by CHIR in d4TCF (based on

adjusted p -value < 0.05 and absolute log-fold-change > 1). Therefore, we consider that approximately 15% of the 166 genes whose expression appears to depend on the presence of β -catenin, do not require the activity of TCF/LEF transcription factors. With our Cut-off values for gene expression fold change we could identify upregulated (4 genes) and downregulated (23 genes) β -catenin-dependent but TCF/LEF-independent changes. These transcriptional changes were validated via RT-qPCR in independent experiments (Figure 3b, 3c, Supplementary Figure 7). We believe that this more cautious formulation is representative of the data, and in line with the criticism raised by the referee.

3. Figure 4b: *HOXC4* and *ZNF503* are presented as examples for supposedly TCF-independent β -Catenin association. However, the genome browser views include a line showing TCF7L2 binding regions from the ENCODE data collection. These appear to be present also at the *HOXC4* and *ZNF503* loci where they coincide with β -Catenin ChIP-seq peak regions. Importantly, β -Catenin binding indeed does seem to be affected in d4TCF cells. In other words, TCF7L2 is present at so-called TCF-independent gene loci and contributes to β -Catenin recruitment. Therefore, occupancy by β -Catenin cannot strictly be called TCF-independent at the *HOXC4* and *ZNF503* loci. In view of this, how many robust and truly TCF-independent β -Catenin binding events are there, which could represent the alternative genomic loci the authors refer to? Which are these loci? It seems there are merely gradual differences where the individual contributions of TCF and non-TCF transcription factors are subject to shifting weights with respect to their roles in recruitment of β -Catenin at different cis-regulatory elements. All of this is perfectly compatible with models of combinatorial and multifactorial control of gene regulation but does not really provide novel and conceptually advanced insights.

We acknowledge a potential contribution of TCF in mediating the binding of β -catenin at these loci. We also now acknowledge the potential requirement of TCF/LEF for transcriptional regulation at these loci (see also below response to point 6). In the manuscript we write (page 16) "On the other hand, other peaks were decreased in size, but still present, such as at the *ZNF503* locus (Figure 4c)". It is possible that this second binding behaviour reflects the fact that β -catenin relies on the presence of TCF/LEF in cooperation with other transcription factors, such as FOXO4. This view is supported by the observation that *ZNF503* and its antisense transcript require TCF/LEF for their transcriptional regulation (Supplementary Table 1). Importantly, also, all β -catenin peaks in d4TCF cells containing a TCF motif also contained a FOXO binding motif (Figure 4f). In this scenario, the removal of TCF/LEF could attenuate, but not fully abolish, β -catenin occupancy at this region, and transcriptional regulation of the affected gene".

4. Figure 4b: *HOXC4* is not among the regulated genes listed in Supplementary Tables 1 and 2, including the GHOST response genes. What is the significance of the association of β -Catenin with this locus? Are there more examples for non-productive β -Catenin binding? Again, I would argue that observations like these question the relevance of the model system.

In the three ChIP-seq experiments we performed we observed a variety of loci potentially occupied by β -catenin in the absence of TCF/LEF. From this observation, it is challenging to define which are the genes whose transcriptional regulation is affected by β -catenin's binding to that specific locus. This difficulty also applies to the peaks located within *HOXC4* locus. As previously mentioned, transcriptional regulation of a gene does not necessarily occur in the proximity of that gene, but it can be mediated by the looping of distant enhancer regions onto promoter regions (Tae Hoon Kim et al., Cell, 2007; Chepelev et al., Cell Research, 2012). Is it also relevant to mention that physical occupancy is not necessarily followed by transcriptional regulation. In a recent paradigmatic example, the glucocorticoid receptor was found to bind to $>10,000$ sites on the genome, but to only regulate the expression of a few hundred genes (see work from Timothy Reddy group, Vockley et al., 2016). This observation is surprising, and adds an additional level of complexity to the aim of merging data deriving from RNA-seq and ChIP-seq experiments. We believe that solving this goes beyond the current scope of our study and, at this stage, we only wish to conclude that chromosomal β -catenin occupancy in the absence of TCF/LEF transcription factors exists.

5. How do the authors explain that *HOXC4* is not listed at all in Supplementary Table 3?

The *HOXC4* peaks were annotated as Refseq "intron (NM_014620, intron 1 of 3)" under 'Annotation' & 'Detailed Annotation' whereas 'Gene Name' referred to nearest TSS (in this case NR_030753/MIR615). We thank the reviewer for pointing out the lack of clarity and have updated Supplementary Table 3 accordingly. The annotation now includes both Refseq and Gene Symbol, and headers have been renamed as 'Nearest TSS' where appropriate.

How did they obtain the ChIP-seq tracks for beta-Catenin at this locus?

Coverage tracks were obtained by excluding duplicates, extending read to 200bp then normalizing read counts to library size as Counts Per Millions mapped (CPM).

Likewise, if ZNF503 represents TCF-independent beta-Catenin binding events why does it not appear in the d4TCF spread sheet in Supplementary Table 3?

For d4TCF, experiment 2 and 3 had lower IP efficiencies than experiment 1 which led to the loss of many peaks (see Supplementary Figure 6b-c) as we, for this type of ChIP experiment, use very conservative cutoffs. Nevertheless, the signal tracks fully support replication of these enriched regions as i) signal disappears in beta-catenin-KO cells and ii) signal matches known TCF-binding regions. The peak calling algorithm is a statistical method which takes local background (up to 10kb) into account which becomes relatively high when IP efficiencies are lower. Reliable identification of ChIP peaks in low efficiency samples is a big hurdle for peak finders and we instead opted for stringent cutoffs to limit False Positives and are aware that we have many False Negative peaks. To address this, we have updated Supplementary Table 3 to include peak calls for each individual replicate making it easy to assess the reproducibility of the peaks.

6. Remarkably, ZNF503 and its antisense transcripts turn up as TCF-dependent genes in Supplementary Table 1! In agreement with this, ZNF503 and the antisense transcripts are not among the GHOST response group. Yet, the authors use this locus as an example for TCF-independent beta-Catenin occupancy. Can the authors explain this discrepancy?

To respond to this, please refer to our responses to points 3 and 4, concerning the difficulty in associating ChIP-seq peaks with transcriptionally regulated loci. But we acknowledge the validity of this observation, and we deem as important to mention it in the manuscript. In the discussion section we now write: “On the other hand, other peaks were decreased in size, but still present, such as at the ZNF503 locus (Figure 4c’). It is possible that this second binding behaviour reflects the fact that b-catenin relies on the presence of TCF/LEF in cooperation with other transcription factors, such as FOXO4. This view is supported by the observation that ZNF503 and its antisense transcript require TCF/LEF for their transcriptional regulation (Supplementary Table 1). Importantly, all β -catenin peaks in d4TCF cells contain the TCF and the FOXO binding motifs (Figure 4f). In this scenario, the removal of TCF/LEF could attenuate, but not fully abolish, b-catenin occupancy at this region, and transcriptional regulation of the affected gene.”.

7. Along the same line: While going over the data, the APCDD1 gene caught my attention. This is a fairly well known Wnt/beta-Catenin target gene. Consistent with this, APCDD1 shows up in Figure 2a with a nice CHIR response in WT cells, and it is listed in Supplementary Table 1 as a TCF-dependent gene. However, in Supplementary Table 2 APCDD1 appears among the 90 GHOST response genes. It is also listed as NOT CHIR-regulated in WT! This does not make any sense to me. I did not go over every single entry of the tables but I am getting a bit worried about data muddling. Therefore, I strongly recommend that the authors carefully check the data presented in their tables and figures for correctness, consistency, and plausibility.

As pointed out by the referee, *APCDD1* is significantly differentially expressed in WT (upregulated when CHIR is added, $\log_{2}FC=+1.5$). However, *APCDD1* was also significantly regulated by CHIR in d4TCF cells, but in the opposite direction (downregulated, $\log_{2}FC=-1.3$). For this reason, *APCDD1* is not included in the group of 27 TCF-independent genes, as this group should only include genes “moving” in the same direction as in WT cells (as our interpretation is that their regulation is unaffected by the removal of TCF/LEF). We now make this clearer in the manuscript and specify, at page 9: “We focused on the high-confidence b-catenin-dependent transcriptional changes and asked whether some of these also occurred in d4TCF cells (Figure 3a). We identified a set of 27 b-catenin-dependent genes that are regulated by CHIR in d4TCF (based on adjusted p-value<0.05 and absolute log-fold-change>1). Therefore, we consider that ca. 15% of the 166 genes whose expression appears to depend on the presence of b-catenin, do not to require the activity of TCF/LEF transcription factors. With our Cut-off values for gene expression, fold change, we could identify upregulated (4 genes) and downregulated (23 genes) b-catenin-dependent but TCF/LEF-independent changes. These transcriptional changes were validated via RT-qPCR in several independent experiments (Figure 3b, 3c, Supplementary Figure 7).”. We have also added and explicative note within Supplementary Table 1 in reference to *APCDD1* behavior.

Nevertheless, *APCDD1* remained in the analysis pipeline for our unbiased downstream analyses (therefore appearing as a TCF-independent “GHOST” gene, Supplementary Table 2). As previously mentioned, this is explained by the fact that, in the absence of TCF/LEF factors, *APCDD1* is regulated dependently on b-catenin in an opposite way, i.e. it is upregulated in WT and

downregulated in d4TCF cells. We have now positively confirmed that a similar issue is not occurring for other genes and *APCDD1* was the single gene displaying this behavior. *APCDD1* represents therefore an “outlier” that does not undermine the validity of our approach. Additionally, we cannot exclude the possibility that this might underlie a real biological phenomenon: *APCDD1* could behave as canonical Wnt target gene in normal conditions, and as GHOST gene in the absence of TCF/LEF or when the TCF- β -catenin interaction is inhibited. We have added a text note in Supplementary Table 2 beside *APCDD1* acknowledging this possibility. We are extremely grateful to the referee for giving us the opportunity to explain more clearly this apparent contradiction.

Additional issues:

8. Figure 1e, Supplementary Figure 1b,c: The labeling of the x-axes is incomplete and confusing. The current versions suggest that cells alternatively received Wnt3a or the TCF/LEF rescue plasmid. It should be made clear that the TCF/LEF rescue samples were also treated with Wnt3a. In Supplementary Figure 1c why did the LEF1 sample not receive CHIR?

We implemented the suggested change, and made the figure clearer.

9. Figure 2b,c: The legend does not match the panels. It was just carried over from the original version. It needs adaptation and proper description of the volcano plots.

We have corrected figure legends of Figure 2.

10. Figure 4f, Supplementary Figure 6: Again, tick marks are missing from the y-axes.

We added tick marks.

11. Figure 5a: In contradiction to the authors' response to my previous request the Venn diagram has not been amended. It still suggests that 196 genes respond to CHIR solely in d4TCF cells which is in conflict with the manuscript text on page 11/12. The authors need to show the number of genes commonly regulated in WT and d4TCF cells in the intersection of the two circles and specify precisely how many of the 196 genes are exclusive to the d4TCF cells.

We now clarify this in the figure 5, by making clear that 196 is the total number of genes that change their expression in a statistically significant way in d4TCF upon CHIR treatment (dark green set). We also indicate the numbers within the subsets and their intersection (171 in WT only / 60 in the overlap / 136 in d4TCF cell only).

12. Figure 5f, Supplementary Figures 9 and 10: There is a problem with the labeling of the x-axis. The authors need to make clear that all samples represent experiments with d4TCF cells, i. e. add this information to the final two bars in Figures 5f and Supplementary Figure 9, and maybe replace d4TCF with "untreated" or "control" in Supplementary Figure 10.

We applied the suggested changes. They all make figure representation clearer.

3rd Editorial Decision

6th Sep 2018

Thank you for submitting the revised version of your manuscript. I have now looked at the manuscript files and your response to the referee concerns and I am happy to see that all their remaining concerns have been addressed. Before I can go on to officially accept the study, there are a few editorial issues that I need you to address in a final revision.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Authors Name: Claudio Cantù, Konrad Basler

Journal Submitted to: The EMBO Journal

Manuscript Number: EMBOJ-2017-98873

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself.

Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No power calculations were performed for this study. We collected as many samples as possible subject to resource constraints.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No sample has been excluded from the analyses
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Samples were randomized during experiment execution. Several of the in vitro experiments were performed independently by more than one author.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	N/A
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For the RNA-seq differential expression, no formal tests of distributional fit were applied. The community consensus is that the negative binomial, which is used for differential expression inference, is a good fit to RNA-seq counts (Marek Gierlinski et al., Bioinformatics, 2015).
Is there an estimate of variation within each group of data?	For the RNA-seq analysis, the dispersion parameter (estimate of variation) is pooled not only across the multiple conditions, but across genes of similar expression level, according to an empirical-Bayes-like procedure (see edgeR from Mark D Robinson et al., Bioinformatics, 2010).
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>

<http://1degreebio.org>

<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>

<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>

<http://ClinicalTrials.gov>

<http://www.consort-statement.org>

<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>

<http://jij.biochem.sun.ac.za>

http://oba.od.nih.gov/biosecurity/biosecurity_documents.html

<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We provided the details of antibodies purchase and validation in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HEK 293T resulted negative for mycoplasma infection.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	We have provided a detailed table description of gene expression changes (RNAseq) and b-catenin peak (ChIPseq) in the main body of the manuscript. In addition, raw data and metafiles have been deposited at the ArrayExpress database, with the following accession numbers: RNA-seq: E-MTAB-7029; ChIP-seq: E-MTAB-7028.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	RNAseq and ChIPseq raw data and metafiles have been deposited at the ArrayExpress database, with the following accession numbers: RNA-seq: E-MTAB-7029; ChIP-seq: E-MTAB-7028.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	This study does not fall under the dual use restriction
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